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Mutations in fibroblast growth factor receptor 1 cause Kallmann syndrome with a wide spectrum of reproductive phenotypes

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Abstract

Background: Kallmann's syndrome (KS) is a clinically and genetically heterogeneous disorder consisting of idiopathic hypogonadotropic hypogonadism (IHH) and anosmia. Mutations in *KALI* causing the X-linked form of KS have been identified in 10% of all KS patients and consistently result in a severe reproductive phenotype. *KALI* gene encodes for anosmin-1, a key protein involved in olfactory and GnRH neuronal migration through a putative interaction with FGFR1. Heterozygous mutations in the *FGFR1* gene accompanied by a high frequency of cleft palate and other facial dysmorphisms were recently identified in 8% of a large KS cohort, yet the reproductive phenotype of KS patients harboring *FGFR1* mutations has not been described.

Results: One hundred and fifty probands with KS (130 males and 20 females) were studied to determine the frequency and distribution of *FGFR1* mutations and their detailed reproductive phenotypes. Fifteen heterozygous mutations in unrelated probands were identified. Twelve missense mutations (p.R78C, p.V102I, p.D224H, p.G237D, p.R254Q, p.V273M, p.E274G, p.Y339C, p.S346C, p.I538V, p.G703S and p.G703R) were distributed among the first, second and third immunoglobulin-like domains (D1–D3), as well as the tyrosine kinase domain (TKD). The mutations Y339C and S346C are located in exon 8B and code for the isoform FGFR1c. Additionally, two nonsense mutations (p.T585X and p.R622X) were documented in the TKD of the protein. A wide spectrum of reproductive function was observed among KS probands including: (1) a severe phenotype demonstrated by micropallus, cryptorchidism, no pubertal development, undetectable serum gonadotropins and low serum testosterone (T) and inhibin B; (2) partial pubertal development; (3) the fertile eunuch variant of IHH with normal testicular size and active spermatogenesis with a reversal of HH after T therapy. In addition, we found an even wider spectrum of reproductive function within pedigrees carrying an *FGFR1* mutation ranging from IHH to delayed puberty to normal reproductive function (anosmia only or asymptomatic carriers). These observations strongly suggest a role for other genes that modify the phenotype of *FGFR1* mutations.

Conclusion: KS patients and family members carrying an *FGFR1* mutation present a broad spectrum of pubertal development in contrast to the almost uniform severe clinical phenotype described in KS subjects with a *KALI* mutation. Additionally, this report implicates the isoform FGFR1c in the pathogenesis of KS.

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Keywords: *FGFR1* mutation; Kallmann syndrome; Pubertal development; Idiopathic hypogonadotropic hypogonadism

1. Introduction

The developmental biology of GnRH is unique and complex. Unlike most neurons in the CNS, the origins of GnRH

neurons lie outside the central nervous system in the nasal placode of the embryonic nasal prominence. Here, they share a common origin and destiny with the olfactory neurons during embryogenesis. Together, they migrate across the cribiform plate toward the developing olfactory bulb (Schwanzel-Fukuda and Pfaff, 1989; Wray et al., 1989). GnRH neurons subsequently disengage from their olfactory guidance fibers and further migrate to their ultimate destination in the arcuate nucleus of

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the medial basal hypothalamus (Tobet et al., 2001). From there, they extend their axons toward the median eminence and establish a neural network capable of synchronizing the secretion of GnRH in a coordinated and pulsatile fashion in the hypophyso-portal circulation. Disrupting any of these complex developmental events leads to defects in the final GnRH network and, in severe cases, idiopathic hypogonadotropic hypogonadism (IHH).

Kallmann syndrome (KS) is a developmental disorder characterized by IHH and anosmia or hyposmia. Classically, KS patients present with absent puberty. However, partial sexual development can occur in a small subset of KS subjects as evidenced by spontaneous testicular growth in males and some degree of breast development in females, suggesting a spectrum of abnormal endogenous GnRH secretion (Pitteloud et al., 2002). In addition, KS can occasionally be diagnosed during the neonatal period on the basis of cryptorchidism, microphallus and absent postnatal surge of LH, FSH and T (Grumbach, 2005). Both neurologic defects (synkinesia and hearing loss) and non-neurologic defects (renal aplasia, high arched palate, labial or palatine cleft and dental agenesis) occur with variable frequency in KS (Hardelin et al., 1993; Quinton et al., 2001). Most men and women with IHH cannot complete sexual maturation unless treated with GnRH or gonadotropins and require lifelong replacement therapy to maintain sexual maturation (Hoffman and Crowley, 1982). Interestingly, a rare subset of patients with KS can undergo subsequent spontaneous recovery of reproductive function (Pitteloud et al., 2005; Quinton et al., 1999).

Genetically, KS is a heterogeneous disorder with an as yet unexplained male predominance (five times less frequent in women) (Seminara et al., 1998). While most cases appear to occur sporadically, X-linked (*KALI*: OMIM#308700), autosomal dominant (AD; *KALI*: OMIM#147950) and autosomal recessive (AR; *KAL3*: OMIM#244200) modes of inheritance have all been documented.

Mutations in *KALI* cause the X-linked form of KS and account for approximately 10% of KS cases (Franco et al., 1991; Legouis et al., 1991). The study of two probands affected by different contiguous gene syndromes, both including KS and an overlapping chromosomal deletion of 540 kb in 8p11, led to the discovery of the *FGFR1* gene as a cause of the AD form of KS (Dode et al., 2003). *FGFR1* mutations were subsequently found in 10% of unrelated KS probands with a high frequency of mid-line defects including cleft palate and dental agenesis (Pitteloud et al., 2005; Dode et al., 2003; Albuissou et al., 2005; Sato et al., 2004). Unexpectedly, asymptomatic carriers of mutations in *FGFR1* were also identified (Pitteloud et al., 2005; Dode et al., 2003; Albuissou et al., 2005; Sato et al., 2004). However, a detailed reproductive phenotype of patients harboring a *FGFR1* mutation has not yet been described.

Therefore, this study aimed to: (1) identify the localization and frequency of *FGFR1* mutations in a large cohort of KS patients; (2) map these newly identified mutations onto the well-characterized molecular structure of FGFR1; (3) describe the spectrum of reproductive phenotypes in KS patients carrying an *FGFR1* mutation.

2. Methods

2.1. Subjects

2.1.1. KS population

One hundred and fifty probands with KS were included in this study (130 males and 20 females, M:F ratio of 6.5:1). KS patients exhibited: (1) absent or incomplete puberty by age 18 years in otherwise healthy patients; (2) serum testosterone (T) ≤ 100 ng/dL in men or serum estradiol (E₂) ≤ 20 pg/mL in women, associated with low to normal serum gonadotropin levels; (3) normal adrenal and growth hormone axes (as assessed by an insulin tolerance test); (4) normal serum thyroid, prolactin and ferritin concentrations; (5) normal MRIs of the hypothalamic–pituitary region; (6) anosmia or hyposmia. Two KS patients were <12 years old and were diagnosed with KS during the neonatal period.

2.1.2. Family members of KS subjects

When possible, family members were studied.

2.1.3. Controls

The Caucasian control population consisted of 200 healthy volunteers who either were consecutive donors to the Massachusetts General Hospital blood bank ($n = 100$ chromosomes) or were healthy volunteers controls as assessed by history and clinical examination ($n = 300$ chromosomes). In addition, 50 controls from Saudi Arabia were selected from an independently assembled cohort. The study was approved by the Human Research Committee of MGH and subjects provided written informed consent prior to participation.

2.2. Mutation analysis of the *FGFR1* gene

Blood samples were obtained from the probands, family members and controls and DNA was extracted. Sequencing of the coding regions of the *FGFR1* gene (GenBank accession number BC018128) as well as the intron–exon boundaries were performed (Pitteloud et al., 2005). Amplified products were sequenced in both directions using the AmpliTaq Dye Terminator Cycle Sequencing kit and an ABI PRISM 377 DNA sequencer (Perkin-Elmer Corp., Foster City, CA, USA). Subsequent sequence analysis was performed via Mutation Surveyor v2.2 (SoftGenetics, State College, PA, USA) and visual inspection. All sequence variations were found on both strands and confirmed in separate PCR reactions. Nonsense changes resulting in either a truncated protein, frameshift, insertion or deletion were categorized as definite mutations. Nucleotide changes that meet the following criteria were also classified as disease causing mutations: (1) absent from the dbSNP and expressed sequence tags (ESTs); (2) absent in ≥ 200 ethnically matched subjects; (3) evolutionarily conserved across species; (4) segregated appropriately in the family. All genes and proteins are described using the standard nomenclature (Antonarakis, 1998).

2.3. Structural analysis of the effects of KS mutations on *FGFR1* function

The crystal structure of the FGFR1 tyrosine kinase domain [protein data bank identification (PDB ID): 1FGK] (Mohammadi et al., 1996) was used to study the effects of the I538V, Y585X, R622X, G703R and G703S mutations which map to the tyrosine kinase domain of FGFR1. The crystal structure of extracellular ligand binding region of FGFR1c encompassing Ig domains D2 and D3, in complex with FGF2 and heparin oligosaccharide (PDB ID: 1FQ9) (Schlessinger et al., 2000) was used to study the effects of D224H, G237D, R254Q, V273M, E274G, T339C and S346C KS mutations which map to this extracellular portion of FGFR1c.

2.4. Clinical studies

2.4.1. Medical history

All subjects were interviewed regarding their medical history with particular attention to the degree of pubertal development and completeness of sexual

maturation. For male subjects, historical evidence for some degree of spontaneous pubertal development was recorded as previously described (Pitteloud et al., 2002). These criteria included: (1) a marked increase in the number of erections, ejaculations and nocturnal emissions; (2) the occurrence of a growth spurt; (3) initiation of shaving; (4) marked increase in libido. The presence of at least two of these historical points or a testicular volume (TV) ≥ 4 mL in the absence of prior gonadotropin therapy was interpreted as evidence of partial spontaneous puberty. Male KS patients not meeting these criteria were defined as having absent puberty. For female subjects, those who lacked breast development by age 14 years and/or did not initiate menses by age 15 years were defined as having absent puberty. Partial puberty was defined as the historical evidence of some degree of spontaneous breast development or growth spurt in the absence of prior sex steroid therapy. In addition, detailed histories of associated non-reproductive phenotypes (i.e. dental agenesis, cleft lip/palate, hearing loss, color blindness ...) were taken.

2.4.2. Family history

Detailed family histories were also recorded. If KS or partial phenotypes such as isolated anosmia, delayed puberty or midline facial defects were present in family members of the proband, the pedigree was classified as familial. The remaining cases were classified as sporadic.

2.4.3. Physical examination

All subjects had measurements of height, weight, eunuchoidal proportions (arm span versus height) and Tanner staging for pubic and axillary hair. Female subjects had Tanner staging for breast development and had uterine and ovarian assessments via palpation. Male subjects were assessed for phallus length [stretched penile length < 2.5 cm = microphallus] and testicular volume (via Prader Orchidometer).

2.4.4. Olfactory acuity

In addition to historical report, olfaction was assessed when possible with formal, quantitative smell testing (the University of Pennsylvania Smell Identification Test (UPSIT, Sensonics Inc., Haddon Hts, NJ, USA) (Doty et al., 1985). A normative range was established on 1819 men and women between 15 and 50 years old (Doty et al., 1985). A normal sense of smell was considered as any score ≥ 5 th percentile.

2.4.5. Baseline neuroendocrine evaluation

When possible, each affected proband was admitted to the MGH General Clinical Research Center for a 12-h frequent sampling study to assess LH pulsatility (q10'). Pulsatile hormone secretion was analyzed using a modification of the Santen and Bardin method (Hayes et al., 1999). Subsequently, subjects were classified according to LH secretion pattern: (1) *apulsatile* defined as complete absence of LH pulses; (2) *low amplitude* defined as a normal LH pulse frequency yet a mean amplitude > 2 S.D. below the mean for normal controls (Spratt et al., 1988); (3) *low frequency* defined as a normal mean LH pulse amplitude (within ± 2 S.D.), but a LH pulse frequency > 2 S.D. below the mean for normal controls; (4) *low frequency and low amplitude*. For each overnight study, a study pool was created from equal aliquots of each of the Q10' samples and was assayed for FSH, T, E₂ and I_B. In those subjects who could provide one, semen samples were collected using standardized methods and were analyzed according to standard WHO criteria.

2.4.6. Hormone assays

Serum LH and FSH concentrations were determined by microparticle enzyme immunoassay (MEIA) using the automated Abbott AxSYM system (Abbott Laboratories, Chicago, IL, USA). The Second International Reference Preparation was used as the reference standard. The assay sensitivity for both LH and FSH was 1.6 IU/L, the intraassay coefficient of variation (CV) for LH and FSH were < 7 and $< 6\%$, respectively. The interassay CVs were $< 7.4\%$ for both hormones. Serum T concentrations were measured using the DPC Coat-A-Count RIA kit (Diagnostic Products Corp., Los Angeles, CA, USA) which had an intraassay and interassay CV of less than 10%. I_B was measured using a commercially available (Serotec, Oxford, UK) double-antibody enzyme-linked immunosorbent assay. In our use, the clinical detection limit of this assay is 50 pg/mL, with a CV of 4–6% within plate and 15–18% between plates.

3. Results

3.1. Molecular analysis of *FGFR1* gene

Sequencing of the coding regions of the *FGFR1* gene in all 150 unrelated KS probands revealed the presence of 15 rare sequence variants in the *FGFR1* gene. Thirteen of them resulted in missense mutations whereas two nucleotide changes (c.1755 C>A and c.1864 C>T) led to two stop codons (p.Y585X and p.R622X). Importantly, p.Y339C and p.S346C residues are encoded by the alternative exon 8B. This exon is specific for FGFR1c and not FGFR1b isoform. The p.R622X has been previously reported as a case report (Table 2 and Figs. 1 and 2) (Pitteloud et al., 2005). All these variants segregated appropriately within the pedigrees (Fig. 1), were not detected in 400 chromosomes from healthy controls, and were highly conserved across species and within the family of FGFRs (Fig. 2).

We also found one previously described polymorphism (SNPs) (dbPM) in our patient population but not in our Caucasian controls (Table 3). In addition, we report three novel SNPs in the coding sequence of the *FGFR1* gene. The details of these variants and their frequency in both the KS cohort and 200 Caucasian controls are shown in Table 3.

3.2. Structural analyses suggest that *FGFR1c* KS mutations will cause receptor loss of function

From the 14 KS mutations identified in this report, 12 localize to the structurally well-characterized portions of FGFR1, including the extracellular D2 and D3, the interconnecting D2–D3 linker which are necessary and sufficient for ligand binding, as well as intracellular tyrosine kinase domain whose activation via tyrosine phosphorylation is required for the initiation of intracellular signaling cascade. The p.D224H and p.G237D mutations affect residues in receptor D2 domain and support the current thinking that KS is a manifestation of FGFR1 loss of function. In the crystal structure, the D224H mutation maps onto the loop region connecting the β E and β F strands and therefore would not affect ligand binding as this region is not at or near the primary FGF–FGFR binding interface (Fig. 3B). Instead, this mutation will lead to loss of FGFR signaling by a completely novel mechanism. In the structure, D224 forms two strong salt bridges with R202 (located in the loop between β C' and β D strands), and also forms a hydrogen bond with backbone amid nitrogen of V221 within the β E– β F loop region (Fig. 3B). These intramolecular bonds facilitate the conformation of the β E– β F as well as the β C'– β E loop regions which in the 2:2:2 FGF–FGFR–HS dimer engage the FGF ligand at the secondary FGF–FGFR interface (Fig. 3B). The D224H mutation would destabilize the conformation of these two loops region thereby reducing the ability of FGFR to undergo ligand and heparin/HS induced dimerization. Importantly, the D224H represents the first example of disease causing FGFR mutations where pathogenesis results specifically from the abrogation of contacts between FGF and FGFR at the secondary FGF–FGFR interaction site. The G237D mutation maps to the tight turn between the strands β F and β G where it makes several intramolecular hydrogen bonds which contribute

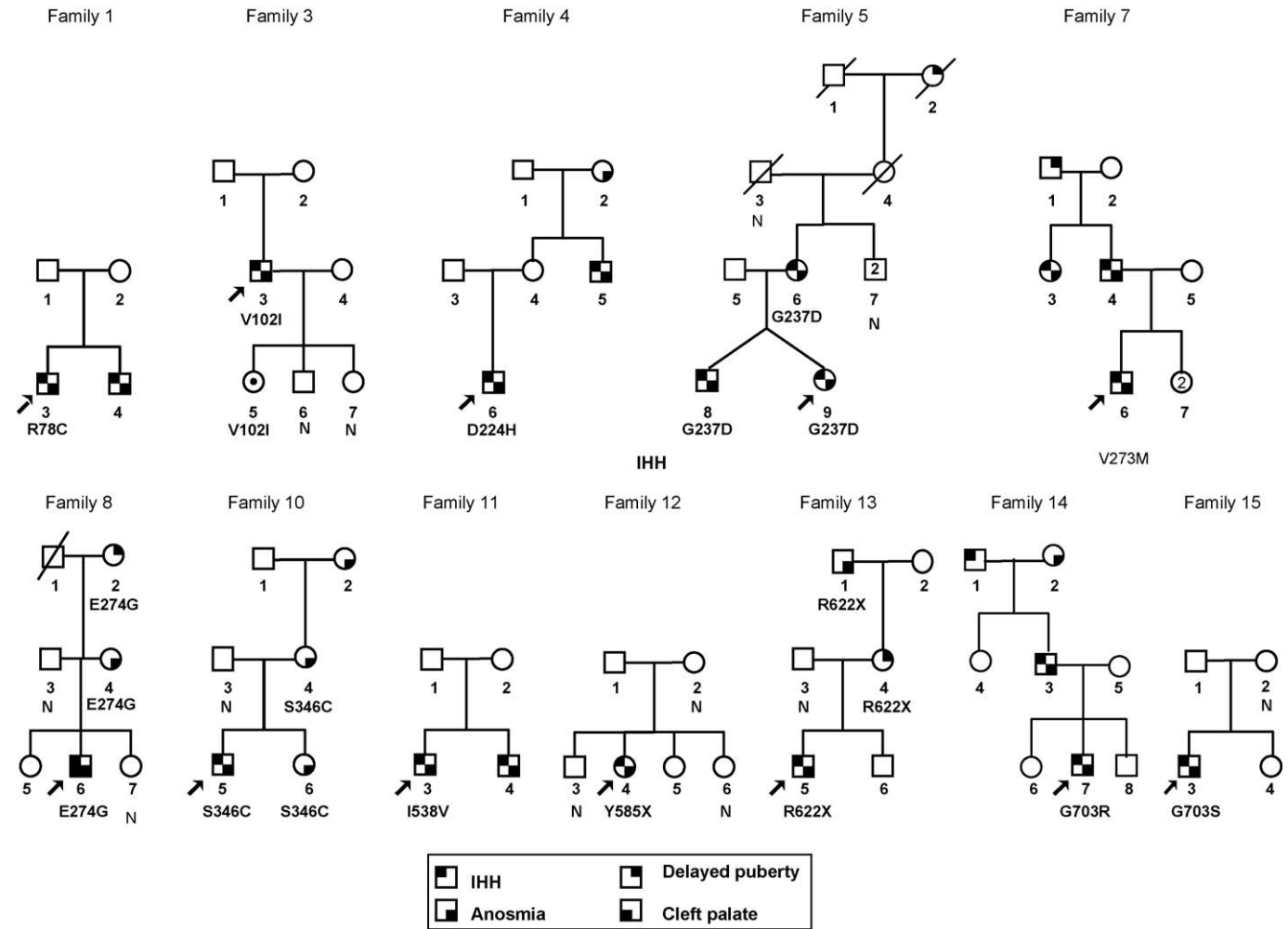


Fig. 1. Segregation of the *FGFR1* mutations in 12 pedigrees with KS. The proband is identified by the arrow. Circles denote females and squares denote males. Phenotypes are as described in the text.

to the proper formation of this turn (Fig 3A). Due to spatial constraints, any residue with side chain such as aspartic acid would introduce steric clashes at this region, and thus is incompatible with the formation of this tight turn. Therefore, the G237D mutation is expected to destabilize D2 folding and ultimately translating in the reduced ligand binding capacity of the mutated receptor. Indeed, we have recently shown that a G237S mutation, identified in patients with normosmic IHH, inhibits refolding of FGFR1c ectodomain *in vitro* (Pitteloud et al., 2006).

The p.R254Q is located in the linker between D2 and D3 and does not seem to play any role in ligand binding. R254 makes two weak solvent-exposed hydrogen bonds with side chain of S350 and the backbone carbonyl oxygen atom of L349 (Fig. 3D). However, these hydrogen bonds seem to play only a subtle role if any in facilitating the structural integrity of FGFR. Based on these structural observation, we propose R254 maybe involved in interaction with another cell surface protein such as HSPG core region or even possibly anosmin-1, the affected protein in KS patients with a *KAL1* mutation.

The p.V273M, p.E274G, p.Y339C and p.S346C substitutions map to D3 domain of the receptor. V273, E274 and Y339 clearly play a role in maintaining the structural integrity of D3. The side

chain of V273 (located on β B strand) points into the hydrophobic interior of D3 and is surrounded by other hydrophobic residues in the D3 core (Fig. 3C). Methionine has a much bulkier hydrophobic side chain and therefore is expected to destabilize the D3 fold by way of steric conflicts with other hydrophobic residues at the end of D3 (Fig. 3C). The E274G mutation also maps to the β B strand. The substitution of E274 with glycine will reduce the ability of the β B strand to form because glycine is a well-known residue unfavorable to β stranding. Y339 residue on the β F strand also participates in the formation of the hydrophobic core at the membrane proximal end of D3 (Fig. 3C). In addition, the phenolic hydroxyl group of this tyrosine makes a hydrogen bond with the backbone carbonyl oxygen atom of D335 located in the loop between β E and β F strand (Fig. 3C). This hydrogen bond plays an important role in facilitating the conformation of the β E– β F loop region and provide a key constituent to the hydrophobic core. Therefore, Y339C substitution is also detrimental to the D3 folding. The destabilizing effects of these mutations on D3 folding should discourage the formation of conserved disulfide bridge between C277 and C341, which define D3 as an Ig domain. As a result, these cysteines should become solvent-exposed and thereby engage in disulfide bridg-

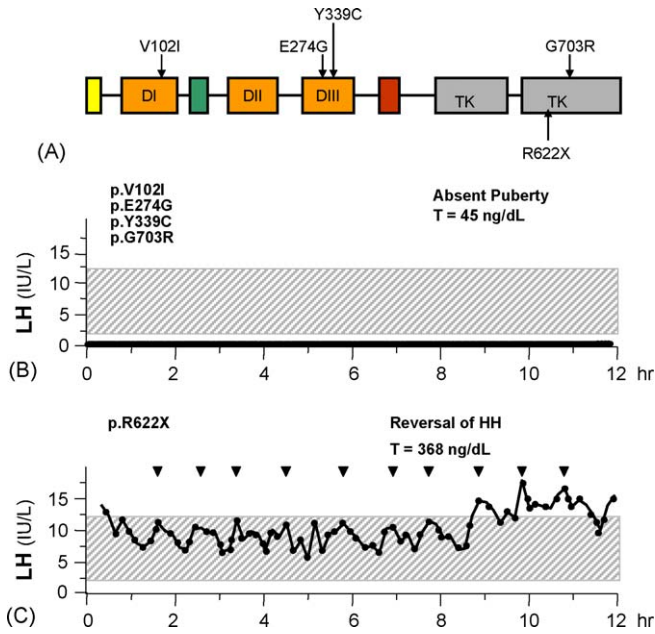


Fig. 2. Genotype–phenotype correlation in five KS probands with an *FGFR1* mutation (A) who underwent neuroendocrine assessment (B and C). An overnight 10 min sampling study for LH depicts endogenous LH secretion. Inverted triangles indicate LH pulses and the normal range of serum LH is shaded.

ing between receptors. This is reminiscent of the mechanism by which gain-of-function mutations activate *FGFR2c* in craniosynostosis syndromes. Indeed, Y339C and S346C mutations are analogous to the constitutively activating Y340C and S347C mutations in *FGFR2c* which cause Crouzon syndrome. However, given the initial genetic finding by Hardelin and colleagues (Dode et al., 2003), and our recent biochemical data showing that KS results from an impairment in *FGFR1c* function (Pitteloud et al., 2006), we believe that ultimately the Y339C and S346C also represent loss-of-function mutations. We suggest that these two mutations interfere with proper folding and maturation of the affected *FGFR1* and lead to an overall decrease in cell surface expression of *FGFR1* and consequently reduced *FGFR1* signaling.

The p.I538V, p.Y585X, p.R622X, p.G703R and p.G703S affect residues which are located in the tyrosine kinase domain of *FGFR1*. The p.Y585X nonsense mutation maps onto the loop region between α D and α E helices, referred to as kinase insert region. The p.R622X nonsense maps onto the catalytic loop of the kinase in the larger C-terminal lobe of kinase domain. Each of these nonsense mutations will delete a major portion of the catalytic TK domain. Alternatively, these mutations may cause haploinsufficiency, as mRNAs with premature termination codons can be targeted to nonsense-mediated decay (Maquat and Carmichael, 2001). I538 is the last residue of α C helix in the N-terminal lobe of kinase domain (Fig. 3E). The aliphatic side chain engages in hydrophobic contact with the F642 in the DFG motif of the kinase domain. This hydrophobic contact is important as it properly positions the F642 side chain such that the ATP binding cleft between N- and C-terminal lobes of the kinase remains accessible for ATP binding (Fig. 3E). The I538V

substitution should reduce the strength of the hydrophobic contact with F642, which could increase the K_m for ATP and thus manifest a reduction in kinase activity of *FGFR1*. It is also noteworthy that I538 is situated approximately at the pivot point upon which N-terminal lobe of kinase domains hinges when the kinase transits from an unphosphorylated state towards the tyrosine phosphorylated activated state. Therefore, the I538V mutation could also discourage this transition. The G703R and G703S mutations map to the loop region between the α F and α G helices in the C-terminal lobe of the kinase domain (Fig. 3F). The G703 residue plays a role in stabilizing the conformation of this loop region, which is also engaged in hydrogen bonds with K665 located at the C-terminal end of the activation loop of the kinase domain (Fig. 3F). Therefore, destabilization of the α F– α G loop conformation due to the G703R/S mutation could negatively impact the conformation of activation loop and result in lowered kinase activity.

The p.R78C and p.V102I mutations map to D1, a region for which currently no structural information is available. Both ligand binding and crystallographic data have clearly demonstrated that D1 is dispensable for ligand binding. In fact, cumulative evidence suggest that D1 interacts with the ligand binding D2–D3 portion of *FGFR* to keep the receptor in an autoinhibited state. Understanding the effects of these two KS mutations on *FGFR* function will require the determination of the crystal structure of possibly the unliganded extracellular region.

3.3. Phenotypes of KS subjects with an *FGFR1* mutation

FGFR1 mutations were identified in 15/150 unrelated KS probands (Tables 1 and 2) including 9/75 familial cases and 6/75 sporadic cases. We identified 13 males and 2 females with KS carrying an *FGFR1* mutation. While the sample size for the female KS cohort is small ($n=20$), the frequency of *FGFR1* mutations appear similar to that of males (approximately 10%). By definition, all subjects presented with IHH and anosmia ($n=14$) or hyposmia ($n=1$). The formal quantitative smell test was conducted in 7/15 KS probands and confirmed the history of anosmia/hyposmia.

3.3.1. Spectrum of reproductive phenotypes in KS probands with an *FGFR1* mutation

The study of KS probands with an *FGFR1* mutation revealed a surprisingly wide spectrum of reproductive phenotypes. The majority of male KS probands ($n=10$) had no sign of spontaneous pubertal development, as evidenced by infantile testes and a high frequency of cryptorchidism and microphallus (60 and 30%, respectively) (Table 1). One KS subject (#2-03) was born with retractile testes and microphallus. He was diagnosed with KS as it was documented that he failed to activate the HPG axis during the neonatal window and also had congenital anosmia. In addition, two KS females displayed absent puberty, as evidenced by lack of spontaneous breast development and primary amenorrhea. In concordance with this severe clinical presentation, these KS subjects had undetectable gonadotropins, hypogonadal T/E₂ and low/undetectable I_B levels (Table 1). Furthermore, four of these subjects, with no prior pubertal development, underwent a

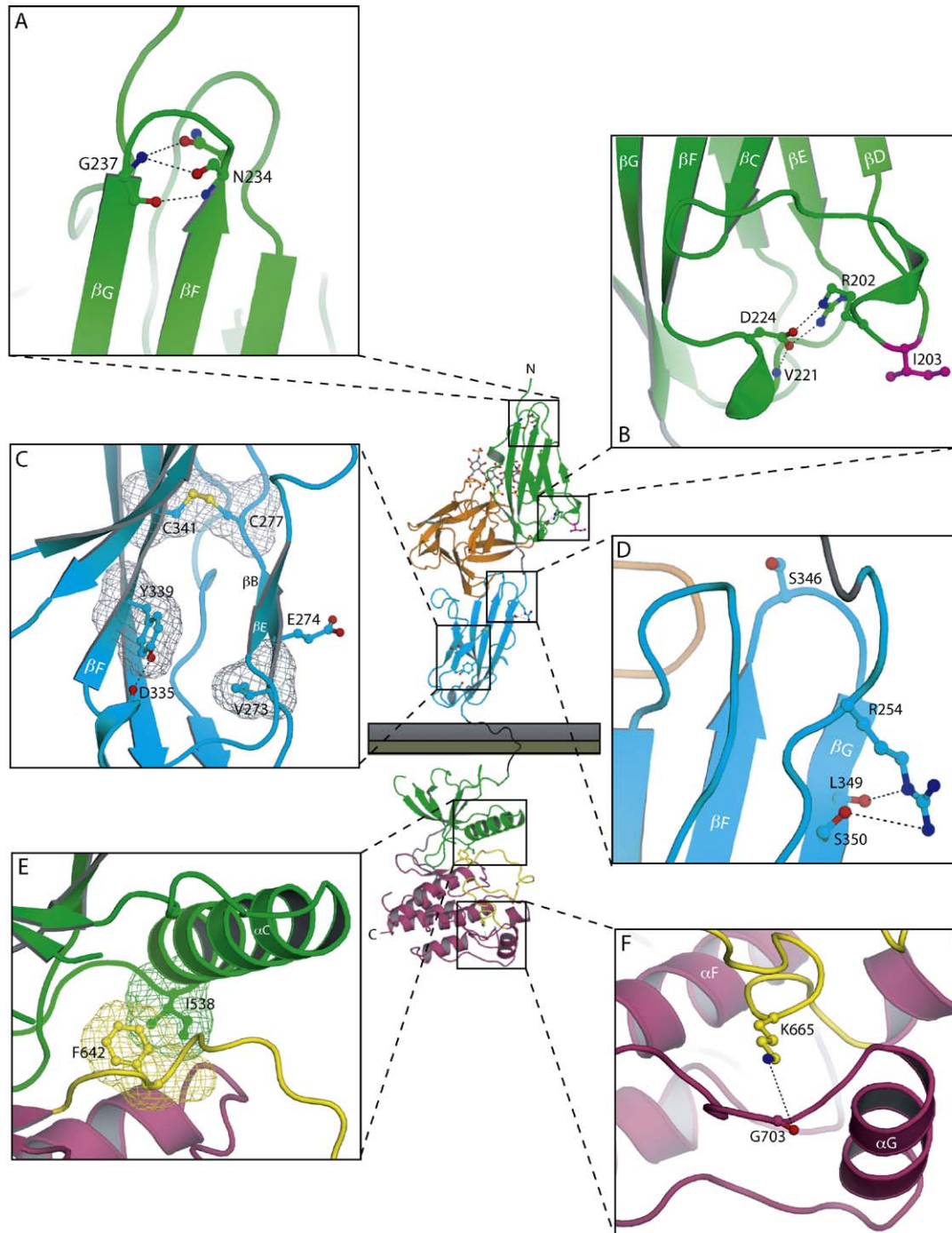


Fig. 3. Analysis of KS mutations in FGFR1c gene in the light of the available molecular structures suggests that these mutations should impair the activity of FGFR1c. The various KS are mapped onto the ribbon diagram of FGF2–FGFR1c–heparin complex, and FGFR1 kinase domain crystal structures. FGF is colored orange and the extracellular ligand binding region of FGFR is colored as follows: D2 in green, D3 in cyan, D2–D3 linker in grey. The coloring of the intracellular tyrosine kinase domain is as follows: the N-terminal lobe of kinase in green, the C-terminal lobe in purple, the activation loop is in yellow and the kinase hinge region is colored grey. Note that ATP (not shown) binds in the cleft between the N-lobe and C-lobe of the kinase domain. Only relevant β strands and α helices of FGFR1c are labeled. Panels A through F, show close-up view of the region where the affected receptor residues are located. In each panel, in addition to the mutated residue, other relevant receptor residues are shown as ball-and-sticks. In Panel B, we also show I203 (colored in purple), which is one of the key residues of FGFR1 that participates in binding of second FGF in the 2:2 FGF–FGFR dimer. Therefore, the D224H mutation should affect the conformation of $\beta C'$ – βD loop, onto which I203 is situated and negatively impact the ability of receptor to undergo ligand- and HS-induced dimerization. To indicate that side chains of V273 and Y339 point into the hydrophobic core of D3 and thus contribute to tertiary fold of D3, in panel C, the surface of these two residues as well as the conserved cysteines of D3 (C277 and C341) are shown as grey mesh. Note that in panel E, the surface of I538 and F642 are also shown as mesh to indicate that these are in close hydrophobic contact with each other. Note that in the fully phosphorylated, activated FGFR kinase domain, the hydrophobic interaction between I538 and F642 repositions F642 such that ATP binding cleft becomes fully accessible for ATP binding. Atom coloring is as follows: nitrogen in blue, oxygen in red, and sulfur in yellow. Hydrogen bonds are shown as dashed lines. Letters N and C denote the N- and C-termini of FGFR1c, respectively. The membrane bi-layer is represented as a grey colored rectangle.

Table 1
Clinical and biochemical features of KS probands with an *FGFR1* mutation

S#	Mutation	Sex	Inheritance	Cryptorchidism	Microphallus	TV (mL)	Breast development	Puberty	T (ng/mL)	E ₂ (pmol/L)	LH (IU/L)	FSH (IU/L)	InhB (pg/mL)
1-03	R78C	M	Familial	Unilat	No	3	NA	Absent	64		<1.6	<1.6	
2-03	R78C	M	Sporadic	Bilat	Yes	2	NA	NA	<4		<1.6	<1.6	<15
3-03	V102I	M	Sporadic	Bilat	Yes	2	NA	Absent	<4		<1.6	<1.6	
4-06	D224H	M	Familial	Bilat	Yes	1	NA	Absent			<1.6	<1.6	14
5-09	G237D	F	Familial	NA	NA	NA	None	Absent		<20	<1.6	<1.6	
6-03	R254Q	M	Sporadic	No	No	2	NA	Absent			<1.6	<1.6	<10
7-06	V273M	M	Familial	No	No	1	NA	Absent	25		<1.6	<1.6	68
8-06	E274G	M	Familial	Bilat	Yes	1	NA	Absent	14		<1.6	<1.6	21
9-03	Y339C	M	Sporadic	No	No	3	NA	Absent	16		<1.6	<1.6	
10-05	S346C	M	Familial	No	No	4	NA	Partial	86		3.5	6	
11-03	I538V	M	Familial	Bilat	No	1	NA	Absent	85		<1.6	<1.6	<15
12-04	T585X	F	Sporadic	NA	NA	NA	None	Absent		47	<1.6	<1.6	
13-05*	R622X	M	Familial	No	No	7	NA	Reversal	17		<1.6	<1.6	53
14-07	G703R	M	Familial	Unilat	No	2	NA	Absent	40		<1.6	4.7	
15-03	G703S	M	Sporadic	Unilat	No	1	NA	Absent	102		<1.6	<1.6	14

12-h frequent sampling for LH. These detailed neuroendocrine evaluations revealed not only apulsatile LH secretion, but levels which were below the assay limit of detection (Fig. 2B). In contrast to these findings, two male probands with KS (#10-05 and 13-05) presented with some degree of spontaneous testicular growth (TV 4 and 7 mL) and no history of cryptorchidism or microphallus (Table 1 and Fig. 1). Notably, one of these men (13-05) harboring a R622X mutation underwent a unique reversal of HH indicated by a normal LH secretion pattern (Fig. 2C), sustained normal serum T levels and active spermatogenesis after 4 years of androgen therapy. A detailed description of this case has been previously reported (Pitteloud et al., 2005).

3.3.2. Variable expressivity of reproductive and non-reproductive phenotypes within families harboring the same *FGFR1* mutation

In pedigree #5 (G237D), the Caucasian mother (5-06) exhibited a severe form of KS: complete absence of puberty and anosmia (score = 9/40). She underwent gonadotropin therapy and conceived fraternal twins (5-09 and 5-08). Both children presented with a severe form of KS and occulo-motor apraxia. The female proband (5-09) had eight missing teeth (a phenotype previously described in patients with an *FGFR1* mutation) (Dode et al., 2003) and the brother had a history of one missing tooth, bilateral cryptorchidism and synkinesia. Interestingly, the deceased maternal great grandmother (5-02) had a history of delayed puberty (menarche at age 20 years). Although no DNA was available for molecular analysis, this family member may well have carried the same *FGFR1* mutation.

In pedigree #8 (E274G), the Caucasian proband (#8-06) had KS with absent puberty, anosmia (score = 6/40), bilateral cryptorchidism, microphallus, cleft lip and synkinesia. The mother (#8-03) had congenital anosmia (score = 9/40) with normal puberty and regular menses while the proband's maternal grandmother (#8-02) had a history of delayed puberty with menarche at age 15 years.

In pedigree #10 (S346C), the Caucasian proband (10-05) presented with KS. He had congenital anosmia (score = 0/40), partial puberty (TV = 4 mL), detectable serum gonadotropins, a normal MRI of the olfactory system and severe osteoporosis. His sister (10-06) and mother (10-04), both harboring the same mutation, underwent normal pubertal development but had congenital anosmia (by report). Although no molecular analysis was performed on the maternal grandmother (10-02), she was also anosmic (by report).

In pedigree #13, the Caucasian KS proband (13-05) had partial puberty and a subsequent reversal of HH. The mother (13-04) had delayed puberty (age of menarche 15 years) and the maternal grandfather (13-01) had anosmia only. Interestingly, all harbor the same *FGFR1* mutation. This pedigree has been previously reported in greater detail (Pitteloud et al., 2005).

In addition, variable reproductive phenotypes within pedigrees were also identified in pedigrees #4, 7 and 14 but molecular analysis could not be performed in other family members (Fig. 1).

Table 2
Mutations in *FGFR1*: genes and functional characteristics

Proband	Nucleotide change	AA change	Position	Functional domain	Novel mutation
1-03	c.232 C>G	p.R78C	Exon 3	D1	Yes
2-03	c.232 C>G	p.R78C	Exon 3	D1	Yes
3-03	c.304 G>A	p.V102I	Exon 3	D1	No
4-06	c.670 G>C	p.D224H	Exon 6	D2	Yes
5-09	c.710 G>A	p.G237D	Exon 6	D2	Yes
6-03	c.761 G>A	p.R254Q	Exon 7	Linker D1–D2	Yes
7-06	c.817 G>A	p.V273M	Exon 7	D3	No
8-06	c.821 G>A	p.E274G	Exon 7	D3	Yes
9-03	c.1016 A>G	p.Y339C	Exon 8B	D3	Yes
10-05	c.1037 C>G	p.S346C	Exon 8B	D3	Yes
11-03	c.1612 A>G	p.I538V	Exon 12	TK	Yes
12-04	c.1755 C>A	p.Y585X	Exon 13	TK	Yes
13-05*	c.1864 C>T	p.R622X	Exon 14	TK	No
14-07	c.2107 G>C	p.G703R	Exon 16	TK	Yes
15-03	c.2107 G>A	p.G703S	Exon 16	TK	Yes

4. Discussion

The *FGFR1* gene consists of 18 exons and encodes one member of the FGFR family, FGFR1. FGFR1 consists of three extracellular Ig-like domains D1–D3, an acid box domain, one transmembrane domain and one tyrosine kinase domain (Fig. 2). Splicing of the carboxy-terminal one-half of D3 results in either the FGFR1b (exon 8A) or FGFR1c (exon 8B) isoforms. These two isoforms differ dramatically in terms of ligand specificity. Dimerization of the receptor is a central step in FGFR1 activation. It requires both the binding to FGF ligand and heparin sulfate (Mohammadi et al., Cytokine & Growth Factor review 2005). In turn, dimerization of the receptor activates several downstream signaling molecules via autophosphorylation in the tyrosine kinase domain of the receptor. FGFR1 is expressed in multiple tissues during development such as skeletal tissue, inner ear and rostral forebrain (Pirvola et al., 2002; Rice et al., 2003) and is required for initial bulb formation (Hebert et al., 2003). Heterozygous deletions, non-sense mutations and missense mutations in the *FGFR1* gene have been shown to underlie one AD form of KS (Pitteloud et al., 2005; Dode et al., 2003; Albuissou et al., 2005; Sato et al., 2004).

This report expands the spectrum of *FGFR1* mutations causing KS identifying 12 novel missense mutations spanning all functional domains of the receptor and implicates the isoform FGFR1c in the etiology of KS. Structural analyses of several of these mutations suggest that they result in receptor loss of function. This is consistent with findings of deletions and stop codons of the *FGFR1* gene underlying some cases of KS.

The 10% prevalence of *FGFR1* mutations in our cohort of KS is consistent with previous reports (Pitteloud et al., 2005; Dode et al., 2003; Albuissou et al., 2005; Sato et al., 2004). Interestingly, of the 35 *FGFR1* mutations reported to date that cause KS (Pitteloud et al., 2005; Dode et al., 2003; Albuissou et al., 2005; Sato et al., 2004), only 4 have been identified in two or more pedigrees (p.R78C, p.V102I, p.V273M and p.R622X). This is in marked contrast with a single gain-of-function mutation in the *FGFR1* gene (p.P252R) underlying several cases of Pfeiffer syndrome, a form of craniosynostosis (Muenke et al., 1994). In addition to identifying and characterizing several novel *FGFR1* mutations, we also identified three synonymous SNPs not previously recorded (Table 3). We have documented the frequency of occurrence of these common variants in our KS cohort and in 200 Caucasians controls. This information could be used in the future to enrich the reproductive genetic profile of each KS patient.

An additional interesting feature in our familial cases is that when genetic screening was performed on other family members, none of the identified mutations were de novo. Several KS probands inherited the *FGFR1* mutation from a parent with a milder phenotype such as anosmia only or delayed puberty (i.e. families 8, 10 and 13). This observation is in contrast to the high penetrance of KS among subjects carrying a *KALI* mutation (Oliveira et al., 2001). Further, the prevalence of *FGFR1* mutations among male and female KS probands was similar in our cohort (10%). However, the mutation was transmitted by the mother, who exhibited a milder or normal reproductive phenotype, in three out of the four familial pedigrees, consis-

Table 3
Prevalence of common sequence variants in the *FGFR1* gene

dbSNP ID	SNP	Amino-acid change	KS cohort frequency (%)	Control frequency (%)
rs2915665	c.345 C>T	S115S	0.9	0
rs2956723	c.2305 C>G	L769V	0	0
New SNP exon 5	c.600 C>T	D200D	0.7	1
New SNP exon 17	c.2262 G>A	L754L	4	1
New SNP exon 9	c.1134 C>T	I378I	0.6	0.5

tent with a prior report demonstrating a similar phenomenon of apparently unaffected mother in *FGFR1* pedigrees (Dode et al., 2003). While additional families need to be studied, it appears mutations could explain part of the male predominance in KS as originally suggested by Dode et al. The biochemical basis for this clinical observation may be supported by the demonstration that anosmin-1 and *FGFR1* interact in vitro (Gonzalez-Martinez et al., 2004) either as a ligand/receptor or co-ligand/receptor motif. Since *KALI* gene resides on X chromosome, females would be expected to have a higher level of *KALI*/anosmin-1 expression than males who are essentially haplo-insufficient for this gene/protein expression. Thus, a higher level of anosmin-1 could partially compensate for any decreases in *FGFR1* function, possibly leading to a milder or normal reproductive phenotype in females carrying an *FGFR1* mutation.

This report documents a remarkably wide spectrum of pubertal development among KS probands carrying an *FGFR1* mutation. In our cohort, the majority of KS probands with *FGFR1* mutations had complete lack of sexual development, a high incidence of cryptorchidism (60%) and microphallus (30%), a uniform lack of GnRH-induced LH secretion, and low/undetectable serum inhibin B levels. Several of these signs may be used as surrogate markers for absence of the HPG axis activity during the late fetal/neonatal window (Pitteloud et al., 2002). In contrast, two male probands exhibited partial puberty with some degree of spontaneous testicular growth (10-05 and 13-05). The latter subject (13-05) was recently described and underwent a unique reversal of his HH following androgen therapy (Pitteloud et al., 2005). These findings are in striking contrast to the almost uniformly severe and highly penetrant reproductive phenotypes described in patients with *KALI* mutations (Pitteloud et al., 2002; Oliveira et al., 2001). Also intriguing is an even wider range of sexual maturation demonstrated within family members harboring the same *FGFR1* mutation including complete absent puberty, partial puberty, delayed puberty, as well as normal reproductive function. This spectrum of reproductive phenotypes within and across families carrying the same *FGFR1* mutation suggests the presence of modifier genes and/or environmental factors.

The X-linked form of KS is thought to result from abnormal GnRH neuronal migration, based on the finding of one fetus with a *KALI* mutation showing arrested GnRH neurons behind the cribiform plate and absent olfactory bulb formation (Bick et al., 1992). Accordingly, male KS subjects with confirmed *KALI* mutations present with severe reproductive phenotypes including absent puberty and high frequency of cryptorchidism and microphallus. In addition, the disease is highly penetrant (Oliveira et al., 2001). Loss-of-function mutations in *FGFR1* are thought to result in a defect in GnRH neuron migration via abnormal olfactory bulb morphogenesis (Dode et al., 2003), somewhat analogous to the *KALI* story. Indeed, while *FGFR1* KO mice are lethal early in development, a conditional KO of *FGFR1* limited to the telencephalon revealed absent olfactory bulbs (Hebert et al., 2003). Consistent with this hypothesis, several MRIs in KS patients carrying an *FGFR1* mutation ((Pitteloud et al., 2005; Sato et al., 2004; Zitzmann and Nieschlag, 2000) and subjects 1-03, 11-03 and 13-05) were confirmed to have aplasia

or hypoplasia of the olfactory bulbs. Furthermore, the majority of our subjects presented with the most complete form of GnRH deficiency as revealed by absent puberty and history of cryptorchidism and microphallus similar to the phenotype seen in KS patients with a *KALI* mutation. However, KS subjects with a milder form of GnRH deficiency and family members with delayed puberty or normal reproductive function harboring an *FGFR1* mutation were also identified, demonstrating variable endogenous GnRH secretion. Therefore, these data indicate that partial or even complete migration of GnRH neurons into the hypothalamus may occur in KS subjects carrying an *FGFR1* mutation. Interestingly, the KS proband (10-05) with a history of partial puberty and anosmia had normal olfactory imaging. This indicates that decreased FGF signaling causing KS is not always associated with anomalies of the olfactory bulbs morphogenesis but may involve earlier step in GnRH neuron differentiation or alter the maturation of GnRH in the hypothalamus. Accordingly, *FGFR1* expression in the mice can be detected in the nasal placode, developing olfactory bulbs, along the GnRH migratory pathways as well as in mature hypothalamic GnRH neurons and their projections (Gill et al., 2004). Further evidence of a broader role of *FGFR1* in GnRH neuron biology comes from mice model with dominant negative *FGFR1* mutants targeted to GnRH neurons (Tsai et al., 2005). These mice demonstrate a 30% reduction in GnRH neuronal population of the hypothalamus and present with delayed puberty and premature ovarian failure consistent with our clinical findings.

In summary, this study adds to the spectrum of loss-of-function mutations causing KS, implicates the isoform *FGFR1c* in the pathogenesis of KS, and confirms the frequency of *FGFR1* mutations to be about 10%. Further, this study demonstrates a wide spectrum of reproductive phenotypes in KS subjects carrying an *FGFR1* mutation indicating that for at least some patients, the defect in GnRH neuron migration is not absolute. Finally, this large cohort of familial cases of KS bearing an *FGFR1* mutation confirms the variability in expressivity of the reproductive and non-reproductive phenotypes among family members carrying the same mutation further suggesting the interaction of *FGFR1* with other genes products.

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